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TWO NEW *DIPLAZIUM* (WOODSIACEAE) SPECIES FROM EAST MALESIA

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Keywords: *Diplazium,* taxonomy, rhizome scales, Sulawesi, Seram,

ABSTRACT

Two new species of *Diplazium* are described from Indonesia: *D. walkeri* Hovenk. from Sulawesi, and *D. murkele* Hovenk. from Seram.

INTRODUCTION

During identification of ferns collected by A.C. Jermy and T. G. Walker in 1969 in the Latimojong Mountains, Sulawesi Selatan (Indonesia), a number of specimens of *Diplazium* (Woodsiaceae) could not be identified with any known species. Comparison with the collections in BM, K and L revealed similarities with *Diplazium mutabile* Hovenk. (Hovenkamp & De Joncheere, 1988), described from a different mountain area on Sulawesi, with *D. furculicolum* Alderw. (Van Alderwerelt van Rosenburgh, 1918), described from Seram, and with a number of collections from Seram identified by Kato et al. (Kato, 1994) as *D. vestitum* C.Presl. With *D. mutabile* the unidentified specimens share a characteristic combination of evanescent basal scales and pinnule rachis ornamentation. The basal scales characteristically disintegrate starting with the outer cell walls (Figure 1A), and the abaxial surfaces of the pinna and pinnule rachis are ornamented with a combination of somewhat fleshy hair-like protrusions and appressed flat scales (Figure 1B). The same rachis indument is found in *D. furculicolum.* and in the Seram collections identified as *D. vestitum*, but accompanied by different types of scales. The scales of *D. furculicolum* have the same thin texture as those of *D. mutabile*, but retain their general outline more strongly; the scales of the Seram "*D. vestitum*" specimens are thick, brown and fleshy, but also appear disintegrated somewhat especially at the margin and the surface of the scale, leaving the exact shape and outline of the scales uncertain. *Diplazium vestitum,* as represented in L by collections from the Philippines and Borneo, differs significantly in having basal scales that are translucent with a dark dentate margin and smooth abaxial rachis surfaces and thereby is clearly different from the Seram specimens.

Notwithstanding the general state of ignorance we are in about the exact species delimitation in Malesian *Diplazium*, the four groups of specimens appear to be sufficiently distinct to warrant the description of two new species. Table 1 provides a synopsis of the major morphological similarities and differences between the four species discussed here.

METHODS

Photographs were taken with a Zeiss Discovery V20 microscope equipped with a Zeiss Axiocam MRC5 camera, and processed with Axiovision software (Axiovision Vs40x64 V. 4.9.10). For scanning electron microscopy (SEM) spores were sputter-coated with gold and examined in a JEOL JSM 5300 microscope (Figure 2A) or coated with c. 5 nm Pt/Pd and observed with a Jeol JSM 7600F FEG-SEM (Figure. 2B, C, D).

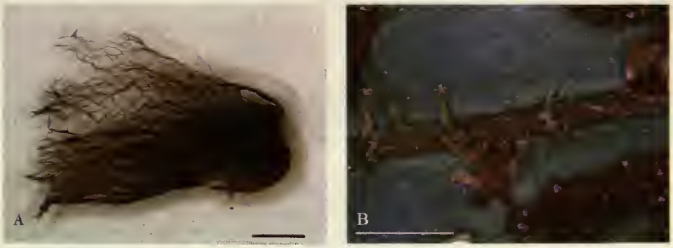

Figure 1. *Diplazium walkeri.*
A: Rhizome scale, showing the fibrous transverse cell walls remaining after the disappearance of the superficial walls (rehydrated). Scale bar 2mm.
B: Abaxial surface of penultimate midrib, showing combination of fleshy hairs and appressed flat scales (*in sicco*). Scale bar 1mm (Walker 12367, BM).

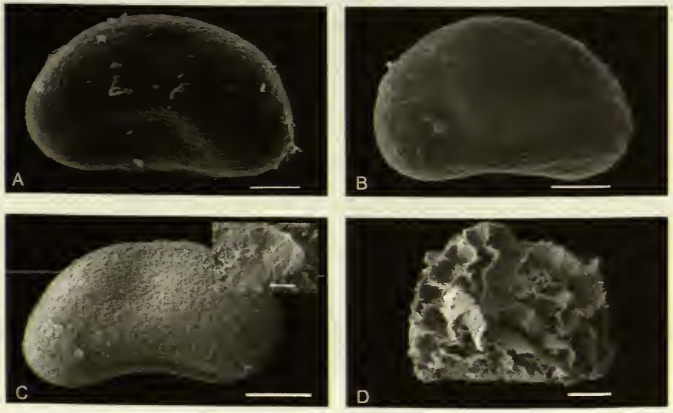

Figure 2. Spores:
A: *Diplazium mutabile*
B: *Diplazium walkeri*
C: *Diplazium murkele*, inset: detail of scaly outer layer
D: *Diplazium furculicolum*
Scale bars 10µm except inset in C 1µm. (Hennipman 5571 (L): A; Kato et al. C 5043 (L): C; C 1124 (L): D, Walker T12373 (BM): B).

Table 1. Diagnostie eharaeters for the four taxa.

	Diplazium mutabile	***Diplazium walkeri***	***Diplazium murkele***	***Diplazium furculicolum***
Rhizome	Ascending or ereet	Aseending or ereet	Aseending to ereet	ereet
Scales on rhizome and base of stipe	Thin, translueent, evaneseent	Thin, translueent, evaneseent	Thiek, spongy, evaneseent?	Thin, translueent, more or less persistent
Reduced pinnae	1-2 (bipinnate speeimens)	3-6	1	1-3
Pinnae length	22-26em	Up to 27em	Up to 36em	7-13em
Pinnae width	6-10cm	Up to 10em	9 -17cm	1-3.5em
Pinnules length	3-5em	e. 5em	5 - 10.5em	6-15mm
Pinnules width	1.5-1.7em	e. 1.5em	1.5 - 3mm	1.5-5mm
Pinnules margin	Serrate-dentate	Deeply ineised, to 2-3mm from midrib	Deeply ineised, to 1-3em from midrib	Crenate or rarely more deeply ineised
Basal pinnules free	2-4 pairs	7-10 pairs	7-9 pairs	0-2 pairs
Lamina apex	Not proliferous	Not proliferous	Not proliferous	Often proliferous
Spore ornamentation	Weakly rugulate	Weakly rugulate	Weakly regulate, surfaee eroding	Ornamented narrow wings

DESCRIPTION OF NEW TAXA

Diplazium walkeri Hovenk., **spec. nov.**

Type: Trevor G. Walker 12373 (holo BM; iso L), Indonesia, Sulawesi Selatan, Enrekang, above the village of Rantelimo, 25km SE of Kalosi, on the south-western slopes of Mt. Rantemario, 1950-2100m, on ridge above northern branch of R. Pasui, 24 November 1969.

Terrestrial fern, rhizome ascending or erect, incl. stipe-base remnants 2-3cm thick; scaly but glabrous in older parts; rhizome scales basifix, periclinal cell walls very thin and quickly disappearing, often leaving the scale disintegrating into a fibrous network, shape, margin, apex of the scales accordingly not observed. Fronds to c. 130cm long; stipes to c. 46cm, sparsely scaly at base with scales similar to those on the rhizome, upwards with papillose multicellular protrusions; lamina bipinnate, to c. 90 by 50cm, widest at c. 1/3-1/5, gradually narrowing from c. halfway up; free pinnae 12-15 pairs, lowest 1-6 somewhat reduced, alternate to subopposite, largest pinnae to 2 cm stalked, to 37 by 18cm; pinnules alternate to opposite, basal 7-10 pairs free, to 5 by 1.5cm, symmetric at base, incised to 1.5-3mm from midrib, apex obtuse to acute, lobes truncate; pinnae and pinnule rachis on the adaxial surface with two flanges interrupted at the vein junctions, on the abaxial surface with flat translucent scales and papillose multicellular protrusions, veins free. Sori diplazioid, on all veins of a vein-group, running from costule to c. 1mm from the margin, spores c. 50 µm long, very lightly rugulate.

Distribution – Indonesia: Sulawesi Selatan (Enrekang District, Latimojong Mountains).

Ecology – From lowland forest to upper montane forest, in deep shade, in ravines or by streams, 1700-2150m.

Etymology - The species is named after Trevor G. Walker (1927-2006), British pteridologist.

Note - *Diplazium walkeri* differs from *D. mutabile* mainly in the more highly dissected lamina. *Diplazium furculicolum* differs from both in a much smaller size, basal scales that retain more of their shape and in much more strongly ornamented spores (Figure 2).

Diplazium murkele Hovenk., **spec. nov.**

Type: M. Kato, K. Ueda & U.W. Mahjar C 1292 (holo L; iso TI, not seen, BO, not seen), Indonesia, Seram, Tehoru & Seram Utara: Manusela National Park: along a trail between Hatumete (seaside) and Maraina (810 m) in Manusela Valley via Hoale Pass (1770m) in Murkele Ridge, 03° 10-14' S, 129° 35-37' E, 18 Nov. 1983.

Terrestrial fern, rhizome ascending or erect, incl. stipe-base remnants 2-3cm thick; scaly but glabrous in older parts; rhizome scales basifix, to c. 10 by 2mm wide, thick, fleshy, margin entire, in the basal part thin, flaccid; apex obtuse. Fronds to c. 120 or longer; stipes to 75cm, naked or sparsely scaly at base with thick brown scales to 10 by 6 mm, lamina bipinnate, to c. 70 by 50cm, widest at c. 1/4, free pinnae 8-12 pairs, lowest pair slightly reduced, alternate to opposite, largest pinnae to 1.5cm stalked, to 37 by 17cm; pinnules alternate to opposite, basal 7-9 pairs free, to 8.5 (10.5) by 1.5-2 (3)cm, symmetric at base, incised to 1—3 mm from midrib, apex obtuse to acute, lobes truncate; pinnae and pinnule rachis on the adaxial surface with two flanges interrupted at the vein junctions, on the abaxial surface with flat translucent scales and papillose multicellular protrusions, veins free. Sori diplazioid, on all veins of a vein-group, running from costule to c. 1mm from the margin, spores c. 40µm long when well-

formed, many collapsed or otherwise malformed, perispore with a thin almost smooth, eroded, scaly outer layer uncovering a finely irregularly porous inner layer.
Distribution – Indonesia, Moluccas: Seram.
Ecology – Terrestrial, in deep shade, near brooks, in ravines etc., in lowland to montane forest, disturbed forest, often noted for calcareous areas, lowland to 1350m, possibly higher.
Etymology - The epithet is to be treated as a word in apposition and derives from Murkele Ridge, the location of the type specimen and a number of other specimens.
Note – *Diplazium murkele* differs from *D. walkeri* mainly in the basal scales that are not thin and translucent but thick and fleshy. However, they often also appear to show signs of disintegration, which may explain the scarcity of intact scales in the collected specimens. The lamina is somewhat smaller, and has distinctly fewer reduced pinnae near the basis, the pinnules on the other hand are a bit larger. The outer layer of the spores appears to erode away, leaving only a partial, scaly cover (Figure 2 C).

Additional Specimens Seen:

Diplazium mutabile
Sulawesi. Palolo: *Hennipman, E. 5003* (L) 21 April 1979; Track to Roroka Timbu, Sopu-valley: *Hennipman, E. 5151* (L) 30 April 1979; Sopu-valley: *Hennipman, E. 5571* (L) 23 May 1979; *5587* (L) 1979, *5625* (L) 26 May 1979; *5640* (L); Gumbassa: *Hennipman, E. 5711* (L) 30 May 1979; Rantemario: *Walker, T.G. 11985* (BM) 1 November 1969; *12369* (BM) 24 November 1969.

Diplazium murkele.
Moluccas. Manusela National Park: *Iwatsuki* et al. *C 1027* (L) 12 November 1983; *Kato* et al. *C 801* (L) 11 November 1983, *C 1292* (L) 18 November 1983; *C 1475* (L) 22 November 1983; *C 1773* (L) 29 November 1983, *C 2924* (L) 30 December 1984; *C 3223* (L) 2 January 1985; *C 3553* (L) 3 January 1985, *C 5043* (L) 22 January 1985, *C 12022* (L) 24 July 1986; *C 13799* (L) 26 August 1986; *C 14089* (L) 27 August 1986.

Diplazium walkeri.
Sulawesi. Rantemario: *Walker, T.G. 11958* (BM, L) 1 November 1969; *11959* (BM,L) *11961* (BM,L) *12365* (BM,L) 24 November 1969; *12368* (BM,L) *12373* (BM,L)

Diplazium furculicolum.
New Guinea. Sattelberg: *Clemens 7059* (BM) 10 September 1937; Moluccas. Manusela National Park: *Kato, M* et al. *C 949* (L) 16 November 1983, *C 1602* (L) 25 November 1983; *C 3216* (L) 2 January 1985, *C 6887* (L) 17 February 1985; *C 7437* (L) 20 February 1985, *C 12191* (L) 27 July 1986; *C 12431* (L) 28 July 1986; *C 12582* (L) 31 July 1986; *C 14325* (L) 7 September 1986; *C 14457* (L) 1 September 1986; *C 1124* (L) 17 November 1983; *C 1207* (L) 16 November 1983; *Parris, B.S. 11089* (L) 27 August 1987.

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EFFECT OF TEMPERATURE ON THE VIABILITY, LIFESPAN AND VIGOUR, OF CHLOROPHYLLOUS SPORES OF *OSMUNDASTRUM CINNAMOMEUM* (OSMUNDACEAE)

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ABSTRACT

Short-lived chlorophyllous spores need special treatment to extend their longevity and vigour for *ex situ* conservation. Germination rates, mean longevity and germination times of chlorophyllous spores of the rare fern *Osmundastrum cinnamomeum* were determined to assess their viability, lifespan and vigour at 18–25°C (room temperature, RT), 4°C and -80°C during 96 weeks of storage. Results showed that at the low temperatures, the viability and vigour were significantly maintained, and the lifespan of *O. cinnamomeum* spores was extended. Spores stored at RT had completely lost all ability to germinate within 12 weeks. Some spores retained their viability for more than 96 weeks at 4°C and -80°C. Mean longevities were 6.1, 41.8, and >96 weeks with storage at RT, 4°C and -80°C respectively. The mean spore germination time (MGT), herein regarded as an indicator of vigour, was affected by both storage temperature and time. Spores stored at RT and 4°C began to lose their vigour after three weeks. However, it did not decline at -80°C until 24 weeks of storage. This study demonstrates that low temperature (4°C) and cryopreservation (-80°C) significantly extended the longevity and vigour of chlorophyllous spores of *O. cinnamomeum*.

Keywords: cryopreservation, mean germination time.

INTRODUCTION

As plants are threatened by habitat loss, pollution, invasive species, and other factors, *in situ* conservation is considered as the primary focus for conserving threatened species. *Ex situ* germplasm storage, on the other hand, is an important complementary aspect of plant conservation (Pence, 2004). A germplasm bank, storing living propagules in a small space, can efficiently maintain genetic variability (Dickie et al., 1990; Smith et al., 2003; Pence, 2008). Conservation plans for a number of threatened fern species include propagation from spores (e.g., Estrelles et al., 2001; Lusby et al., 2002; Simabukuro et al., 1998). Spore banks have low financial costs and have been developed for more than 20 years to conserve ferns *ex situ* (Page et al., 1992; Ibars & Estrelles, 2012).

Most fern spores can typically retain their viability for a long period but there is great disparity among species (Okada, 1929). One of the principal features causing such

variation is the presence of chlorophyll pigments. Fern spores are primarily divided into chlorophyllous spores (also called green spores) and non-chlorophyllous spores (non-green spores), depending on whether or not chlorophyll pigments exist in them (Gabriel y Galán & Prada, 2011). Compared with non-chlorophyllous spores, which retain their viability for several months to years, chlorophyllous spores are relatively short-lived, surviving for only a few weeks or months at room temperature (Lloyd & Klekowski, 1970).

Chlorophyllous spores quickly lose their viability because of a lack of desiccation tolerance, a high metabolic rate, and the absence of dormancy (Kato, 1976; Li & Shi, 2014). Therefore they require specialised treatment for long-term conservation in germplasm banks (Ballesteros et al., 2011). It is well documented that the viability of chlorophyllous spores can usually be extended under low temperatures (Ballesteros, 2011; Gabriel y Galán & Prada, 2011). The longevity of chlorophyllous and non-chlorophyllous spores has been studied for over 50 years, and some protocols have been established to optimise spore preservation in fern spore banks (Ibars & Estrelles, 2012). For example, low temperature (-70°C) storage substantially prolonged chlorophyllous spore viability of *Equisetum hyemale* L. for over 16 months (Whittier, 1996), and at -20°C for more than 28 months for *Osmunda regalis* L. (Magrini & Scoppola, 2012). The "viability" of spores has been variously defined, such as the length of time that spores retain their ability to germinate (Raghavan, 1989; Gabriel y Galán & Prada, 2011), or the capacity of a spore to germinate (Qunitanilla et al., 2002). The former one is mutually used with the term "longevity" (e.g., Aragón & Pangua, 2004, Pence, 2004). We herein define "spore longevity" (the lifespan of a spore) to be the period from spore harvest to loss of germination ability as per Windham et al. (1986) and Ballesteros et al. (2011). "Spore viability" is defined as the spore germination ability as indicated by the germination rate (%) (Gabriel y Galán & Prada, 2011).

The quality of spores (spore vigour) is related to the germination rate and ageing during storage. Because spore germination time is prolonged with storage time (Smith & Robinson, 1975; Camloh, 1999), germination time was used to indicate spore vigour in this study.

Osmundastrum cinnamomeum (L.) C.Presl is a temperate fern, usually growing in swamps and widely distributed from Asia to the Americas (Metzgar et al., 2008). However, the population number is low (≤4) and relatively small (<200 mature individuals in the largest population) in Taiwan (Chiu et al., 2013). It was evaluated to be a vulnerable species based on IUCN red list categories and criteria (Wang et al., 2012). The goal of this study was to determine the effect of various storage temperatures on the longevity and vigour of chlorophyllous spores of *O. cinnamomeum*. The results provide useful information for long-term *ex situ* conservation of this rare fern in spore banks.

MATERIALS AND METHODS

Fresh spores of *O. cinnamomeum* were obtained from 12 fertile fronds of six plants growing in a marsh at Tsaopi (24°46'03"N, 121°36'26"E, elev. 809m), Yilan County, northeastern Taiwan, in March 2012. The fertile fronds released spores after being air-dried for three days. Spores were isolated from sporangia and transferred to microtubes (Labcon Screw-Cap Microcentrifuge Tubes, 2.0ml) for dry storage. Microtubes of spores were stored at 18–25°C (room temperature, RT), 4°C or -80°C for up to 96 weeks.

Spores stored at RT, 4°C and -80°C were sown separately onto four sheets of membrane filters (with a pore size of 0.45μm and diameter of 47mm; Gelman Laboratory) after 0 (fresh spores), 1, 2, 3, 4, 8, 12, 24, 48 and 96 weeks of storage. Each sheet was laid on a 4cm thick layer of moist sphagnum moss in a plastic box (114×86×102mm, PHYTATRAY II™, Sigma). All cultures were incubated under white fluorescent illumination at approximately 25–35μmol m^{-2} s^{-1} for 12h d^{-1} at 20 ± 1°C.

Germination refers to the breakage of the spore wall and the appearance of the first rhizoid or gametophyte cell (Lloyd & Klekowski, 1970). To calculate the germination rate (%), 200 spores (50 spores/filter × 4 filters) were randomly observed under a microscope (Leica, Wild M8) every week after sowing until no more germination was detected.

The mean germination time (MGT) was calculated for each replication per treatment according to the equation: $MGT = \Sigma(tn)/(\Sigma n)$, where t is the time in weeks starting from spore sowing and n is the number of spores that had germinated in week t (modified from De Brum & Randi, 2006). The spore lifespan was indicated by the spores' mean longevity calculated by the equation: $ML = \Sigma t_i (p_i - p_{i+1})$, where t_i is the spore storage time (i^{th} week), p_i is the germination rate in the i^{th} week, and p_{i+1} is the germination rate for the next storage time ($i+1^{th}$ week).

Germination rates and mean germination times for each temperature were analysed by a one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test ($p<0.05$) to estimate significant differences of means among different storage times.

RESULTS

All (100%) fresh spores of *O. cinnamomeum* germinated within one week. With RT storage, the spore germination rate remained at almost 100% for four weeks in storage. Then it decreased significantly to nearly 50% after eight weeks in storage, and spores had completely lost viability after 12 weeks in storage. When the storage temperature was lowered to 4°C, spores retained almost all of their viability for 24 weeks in storage. Then it decreased significantly to 69.5% and 3.0% after 48 and 96 weeks of storage, respectively. For spores stored at -80°C, the germination percentage was almost 100% during 48 weeks of storage and still remained at 91.5% even after 96 weeks of storage (Table 1).

The MGT of fresh spores was one week. Under RT storage, the MGT increased significantly to 1.4 weeks after three weeks in storage, and up to 2.0 and 2.1 weeks after four and eight weeks in storage, respectively. The MGT of spores stored at 4°C did not increase within two weeks of storage, then slightly (but significantly) increased to 1.3–1.5 weeks during 3–12 weeks of storage, and again significantly increased to 2.4–2.5 weeks after 24–48 weeks of storage. However MGT was found to have decreased to 2.0 weeks after 96 weeks of storage. The MGT was ≤1.2 weeks when spores were stored at -80°C after 12 weeks in storage, but significantly increased to 2.2 weeks after 24 weeks of storage, then decreased to 1.9 and 1.7 weeks respectively when spores were stored for 48 and 96 weeks (Table 2).

Spores had completely lost their viability after 12 weeks of storage at RT, whereas some viability was retained at 4°C and -80°C storage at the end of the observation period (96 weeks) (Table 2). Thus the lifespan of at least some proportion of spores at 4°C and -80°C was more than 96 weeks. The mean longevity of spores significantly

Table 1. Germination rates of spores of *Osmundastrum cinnamomeum* at different storage times and temperatures

Storage time (weeks)	Germination rate (%)[1,2]		
	Storage temperature		
	RT[3]	4°C	- 80°C
0	100[a]	--	--
1	100[a]	100[a]	100[a]
2	100[a]	100[a]	100[a]
3	100[a]	100[a]	100[a]
4	99.5±1.0[a]	100[a]	100[a]
8	51.5±4.4[b]	100[a]	100[a]
12	0[c]	100[a]	100[a]
24	0[c]	97.0±3.5[a]	96.5±4.7[a]
48	0[c]	69.5±10[b]	98.5±1.9[a]
96	0 [c]	3.0±1.2[c]	91.5±5.3[b]

1) Data are the mean±standard deviation (n=4).
2) Means with the same superscript letter do not significantly differ within a temperature (p<0.05; LSD test).
3) Room temperature of 18–25°C.

Table 2. Mean germination times of spores of *Osmundastrum cinnamomeum* at different storage times and temperatures

Mean germination time (weeks)[1,2]			
Storage time (weeks)	Storage temperature		
	RT[3]	4°C	- 80°C
0	1.0[a]	-[4]	-
1	1.1±0.0[a]	1.1±0.0[a]	1.1±0.0[a]
2	1.1±0.1[a]	1.1±0.0[a]	1.1±0.1[a]
3	1.4±0.2[b]	1.3±0.1[b]	1.1±0.0[a]
4	2.0±0.2[c]	1.3±0.0[b]	1.2±0.1[a]
8	2.1±0.2[c]	1.5±0.0[c]	1.1±0.0[a]
12	--[5]	1.4±0.1[bc]	1.1±0.0[a]
24	--	2.5±0.2[e]	2.2±0.3[d]
48	--	2.4±0.2[e]	1.9±0.1[c]
96	--	2.0± 0.0[d]	1.7±0.2[b]

1) Data are the mean±standard deviation (n=4).
2) Means with the same superscript letter do not significantly differ within a temperature (p<0.05; LSD test).
3) Room temperature of 18−25°C.
4) -, no data.
5) --, spores did not germinate.

differed among the three temperature treatments, with 6.1, 41.8, and >96 weeks at RT, 4°C and -80°C storage, respectively.

DISCUSSION

The viability of chlorophyllous spores under RT conditions is known to be lost rapidly but there are great disparities among species. For example, longevity of chlorophyllous spores of *Equisetum hyemale*, *Osmunda regalis,* and *Matteuccia* sp. are two weeks, ca. one month, and more than one year, respectively (Stokey, 1951; Lloyd & Klekowski, 1970; Lebkuecher, 1997). This study demonstrated that the lifespan of a proportion of spores of *O. cinnamomeum* at RT reached eight weeks, slightly longer than previous records from Japan (1.4–1.8 months) (Okada, 1929). This difference may have resulted from plant genetic variations and/or spore maturity. Fully mature spores usually have greater longevity than do immature ones (Huang et al., 2014). Some physiological mechanisms and compounds, including the respiration rate, water content, catalase content and granules of lipids and lipoproteins, were documented to affect chlorophyllous spore viability (Okada, 1929; Gullvåg, 1969; Olsen & Gullvåg, 1973). The decay of fern spore viability might also result from the loss of some housekeeping substances, such as sugars, amino acids and proteins (Beri & Bir, 1993; Ballesteros et al., 2004). Spore viability is also related to various kinds of sulphur metabolites, such as glutathione (GSH), glutathione disulphide (GSSG), *S*-adenosylmethionine (SAM), and *S*-adenosylhomocysteine (SAH), which are involved in modulating protein activities or other metabolites under stress (Giovanelli et al., 1985; Dixon et al., 2005; Michelet et al., 2005; Loenen, 2006; Roje, 2006). Chang et al. (2013) further documented that GSH/GSSG and SAM/SAH ratios of spores of *O. cinnamomeum* significantly declined as they lost viability. Apparently those physiochemical changes affect the germination rates of *O. cinnamomea* spores and probably are correlated with the loss of viability in other chlorophyllous fern spores.

Chlorophyllous spores tend to germinate quickly because their photosynthetically active chloroplasts can rapidly transform light energy to metabolic activity (Raghavan, 1989). The MGT of chlorophyllous spores at RT is ca. 1.5 days for the Equisetaceae, Osmundaceae, Grammitidaceae, Hymenophyllaceae, *Blechnum nudum* (Labill.) Mett. ex Luerss., *Lomariopsis sorbifolia* (L.) Fée, and some species of *Christiopteris*, *Marginariopsis*, *Matteuccia*, *Onoclea* and *Onocleopsis* (Lloyd & Klekowski, 1970). It is obvious that the germination rate of chlorophyllous spores should be measured at short intervals. Daily observations would provide more accurate data than the weekly observations that were used in this study. Nevertheless, correlations between spore vigour and different storage temperatures were distinguishable.

The results of this study show that the MGT increased before the germination rate declined. However, the MGT of *O. cinnamomeum* spores increased significantly after three weeks of storage at 4°C, then decreased after 96 weeks of storage. A similar situation also occurred with storage at -80°C. The increase and decrease in the MGT may have resulted from the self-regulation of spores in response to stress during storage (Chang et al., 2013). More studies are needed to determine whether the MGT is a good indicator for evaluating the germination vigour of fern spores.

The successful establishment of individuals and populations of a species in a new habitat after spore dispersal depends on how long the spores retain their viability and vigour (Sheffield, 1996; Shorina, 2001; Gabriel y Galán & Prada, 2011). When the spore vigour decreases, subsequent gametophytes may grow abnormally and even

affect subsequent sporophyte production (Smith & Robinson, 1975; Beri & Bir, 1993) and population establishment (Gabriel y Galán & Prada, 2011).

An increase in the storage time results in decline in spore viability and vigour as previously reported for *O. cinnamomeum* and a few other ferns (Smith & Robinson, 1975; Ballesteros et al., 2011, 2012). Evidence was presented that suggests spore age may be partly attributable to the declining ability of spores to synthesise metabolites essential for germination (Gabriel y Galán & Prada, 2011). It is well known that chlorophyllous spores have shorter lifespans compared with non-chlorophyllous spores (Lloyd & Klekowski, 1970). Using low temperature to prolong their lifespan and vigour was documented (Pence, 2000, 2008; Ballesteros et al., 2006, 2011, 2012; Ballesteros, 2011; Gabriel y Galán & Prada, 2011). It was reported that chlorophyllous spores of *Osmunda regalis* and *O. japonica* Thunb. stored at low temperatures (-25 and 4°C) also retained viability for up to 24 and 12 months respectively (Ballesteros et al., 2011; Li & Shi, 2014). This study also showed that spores of *O. cinnamomeum* rapidly lost viability at RT storage, and the lowest temperature of -80°C provided the best conditions to retain their spore viability and vigour. Cryopreservation is, therefore, recommended for the long-term storage of spores of this species, and this procedure may be applicable to other fern spores in terms of *ex situ* conservation.

CONCLUSIONS

Chlorophyllous spores of ferns have shorter lifespans at room temperature storage compared with non-chlorophyllous spores. However, cryogenic temperatures might suppress metabolic activities and effectively extend their longevity and retain the vigour of chlorophyllous spores as shown in this study. It is suggested that cryopreservation is a feasible method for establishing long-term germplasm of the chlorophyllous spores of *Osmundastrum cinnamomeum*.

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PLEOPELTIS ×CERRO-ALTOENSIS (POLYPODIACEAE), A NEW FERN HYBRID FROM ROBINSON CRUSOE ISLAND (JUAN FERNANDEZ ARCHIPELAGO, CHILE)

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Keywords : *Pleopeltis*, hybrid, Polypodiaceae, Juan Fernández, Chile

ABSTRACT

A fern hybrid of the genus *Pleopeltis* was discovered on Robinson Crusoe Island in the Juan Fernández Archipelago, off the coast of Chile, and is described as *P.* ×*cerro-altoensis*. Its putative parents are *P. macrocarpa* and *P. masafuerae*, two species present in the archipelago.

Mots-clés : *Pleopeltis*, hybride, Polypodiaceae, Juan Fernández, Chili

RÉSUMÉ

Un hybride de fougère appartenant au genre *Pleopeltis* a été découvert sur l'île Robinson Crusoë, dans l'archipel Juan Fernández, au large du Chili, et est décrit sous le nom de *P.* ×*cerro-altoensis*. Ses parents probables sont *P. macrocarpa* et *P. masafuerae*, deux espèces présentes dans l'archipel.

Palabras clavas : *Pleopeltis*, híbrido, Polypodiaceae, Juan Fernández, Chile

RESUMEN

Un híbrido de helecho que pertenece al género *Pleopeltis* ha sido descubierto en la isla Robinson Crusoe, en el archipiélago Juan Fernández, a la altura de Chile, y es descrito con el nombre de *P.* ×*cerro-altoensis*. Sus parientes probables son *P. macrocarpa* y *P. masafuerae*, dos especies presentes en el archipiélago.

INTRODUCTION

Since 1997, the first author has undertaken, with the association ROBINSONIA[1], regular expeditions in the Juan Fernández Archipelago, off the coast of Chile, to study

[1] French association registered under Law 1901, on 9th October 2001, involved in the inventory and conservation of the Flora of Juan Fernández Archipelago, in relation with local communities and notably with the *Corporación Nacional Forestal* (CONAF) of Chile.

both the indigenous and the introduced flora of the three islands that constitute this archipelago (Figure 1): the main island, Robinson Crusoe (or Masatierra) and the nearby Santa Clara Island (also called Île aux Chèvres), and the third one, Alejandro Selkirk Island (or Masafuera), located 180 km further west.

During these expeditions, on 29th January 1999, a small isolated population of a polypodiaceous fern was discovered on a cliff of volcanic rocks, at Cerro Alto (Figures 2 & 3) on Robinson Crusoe Island (Masatierra). The presence of peltate scales on both surfaces of the lamina and its axes showed that it belongs to the genus *Pleopeltis* Humb. & Bonpl. ex Willd. (Figures 4 & 7).

We compared this plant with the *Pleopeltis* species known from the area, but our review of botanical literature relating to Juan Fernández (Hemsley, 1884; Johow, 1892-93, 1896; Christensen & Skottsberg, 1920; Skottsberg, 1953; Barrera Moscoso, 1997; Marticorena et al., 1998; Boudrie, 2003; Danton & Perrier, 2006) and to continental Chile (Gay, 1853; Looser, 1961, 1962, 1965-66, 1968; de la Sota, 1967; Marticorena & Rodriguez, 1995; Zuloaga et al., 2008) has shown that it was not recorded in any of the floras or floristic catalogues, nor any description applied to it. Therefore, whatever its identity or origin, we believe that it is a taxon new to the Juan Fernández Archipelago.

Two other species of *Pleopeltis* (previously placed in the genus *Polypodium*) occur in the Juan Fernández Archipelago (Figure 5):

- *Pleopeltis macrocarpa* (Bory ex Willd.) Kaulf., *Berlin. Jahrb. Pharm. Verbundenen Wiss.* 21: 41. 1820.
  Basionym: *Polypodium macrocarpum* Bory ex Willd., *Sp. Pl.* ed. 4, 5: 147. 1810.
  Syn. : *Polypodium lanceolatum* L., *Sp. Pl.* 2: 1802. 1753.
  Distribution: almost pantropical, America (from Central America to Chile and Argentina, Juan Fernández, Galapagos), tropical southern Africa, India.
- *Pleopeltis masafuerae* (Phil.) de la Sota, *Darwiniana* 45(2): 239. 2007.
  Basionym : *Polypodium masafuerae* Phil., *Linnaea* 29: 107. 1857.
  Distribution: Juan Fernández Archipelago (only on Alexander Selkirk Island = Masafuera), Chile (province of Antofagasta); mentioned from southern Peru (Marticorena & Rodriguez, 1995), but not confirmed to our knowledge.

Concerning the identity of *P. masafuerae*, its relationships with several South American *Pleopeltis* such as *P. pinnatifida* (Gillies) Hook. & Grev., *P. pycnocarpa* (C.Chr.) A.R.Sm. and *Polypodium mollendense* Maxon are unresolved (pers. comm. A.R. Smith, 2015). Although the circumscription of *Pleopeltis* has recently been enlarged, based both on molecular and on morphological studies (Otto et al., 2009; Smith & Tejero-Díez, 2014), Neotropical *Pleopeltis* taxa are a taxonomically difficult group, with polyploids and numerous hybrids, and a modern revision is needed to clarify the taxonomy. Tryon & Stolze (1993), treating the Peruvian species (in *Polypodium*), alluded to these complexities.

Because neither of these two species matched the newly discovered plant, which thus might represent a new taxon (Danton et al. 1999), a more careful study of it was undertaken, as described below.

MATERIAL AND METHODS

The material used for this study comprises:

- Fronds collected in the field at Cerro Alto, now pressed and dried herbarium specimens:
  - *Danton B(652)619* (P, SGO, private herb. Ph. Danton)

- *Boudrie 3793* (P)

- Fresh fronds collected at the Botanical Conservatory of Mulhouse, France, where a *Pleopeltis* plant collected by one of us (Ph. D.) at Cerro Alto on Feb. 27, 2002, has been in cultivation since 2002 under the number *CBM 03-301*, and now, extensively propagated. All the herbarium specimens mentioned below are issued from the *CBM 03-301* plant.
  - *Ph. Danton – Mulhouse – 1* (private herb. R. Viane)
  - *Ph. Danton – Mulhouse – 2* (private herb. R. Viane)
  - *Ph. Danton – Mulhouse – 3* (private herb. R. Viane)
  - *Bizot 2905* (private herb. A. Bizot)

Figure 1: Location of the Juan Fernández Archipelago and Robinson Crusoe Island, west of Chile. Map base source : © Google maps, 2015

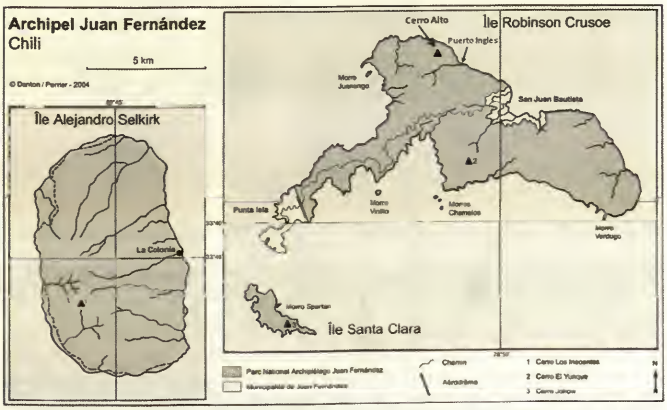

Figure 2: Alexander Selkirk Island (left) and Robinson Crusoe Island (right). © Ph. Danton & Ch. Perrier, 2004

Figure 3: *Pleopeltis* ×*cerro-altoensis* in situ at Cerro Alto. © Photo Ph. Danton, 22/01/1999

Figure 4: *Pleopeltis* ×*cerro-altoensis*. © Ph. Danton, 25/01/1999. a - entire plant; b - portion of adaxial laminar surface; c - abaxial surface of fertile frond; d – peltate-based lanceolate laminar scale; e – peltate-based lanceolate rhizome scale.

Herbier Arch. Juan Fernández n° G(1319)1083
Pleopeltis masafuerae (Phil.) de la Sota
Determ. Philippe DANTON, anno 2011/05/2
Herbier Arch. Juan Fernández n° B(410)366
Pleopeltis macrocarpa (Bory ex Willd.) Kaulf.
Determ. Philippe DANTON, anno 2007/01/27

Figure 5 – Left: *Pleopeltis masafuerae* (Phil.) de la Sota, rocks at the beginning of the *Quebrada Las Casas*, Alejandro Selkirk Island, 19/12/2003, *Danton G(1319)1083*. – Right : *Pleopeltis macrocarpa* (Bory ex Willd.) Kaulf., epiphytic in forest under the *Mirador de Selkirk*, Robinson Crusoe Island, 07/12/1998, *Danton B(410)366*. © Photo Ph. Danton, 26/05/2015.

TYPE
Pleopeltis sp.
B(652)619
Archipel Juan Fernández, île Robinson Crusoe, Cerro Alto, Quebrada Puerto Ingles - Rochers en falaise sur le versant est-sud-est du cerro ≃ 300m
Rec. Ph. Danton 22/01/1999.

Figure 6: *Pleopeltis ×cerro-altoensis* Danton & Boudrie, Cerro Alto, Robinson Crusoe Island, Juan Fernández Archipelago, holotype (SGO). *Danton B(658)619*. © Photo Ph. Danton, 17/11/2014.

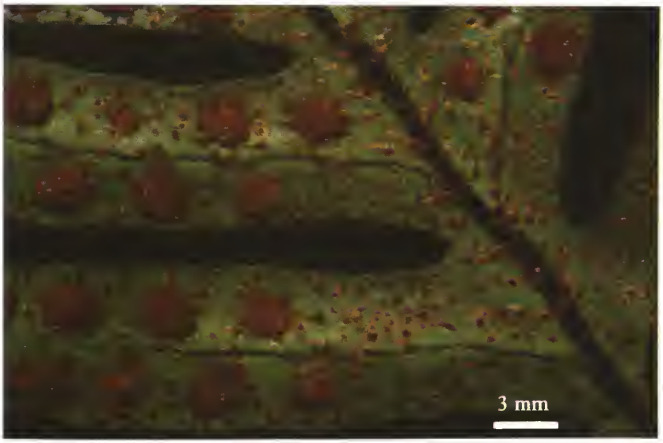

Figure 7: Abaxial laminar surface of *Pleopeltis ×cerro-altoensis*, showing peltate-lanceolate scales and sori (from *Bizot 2905*). © Photo A. Bizot, 8/10/2014.

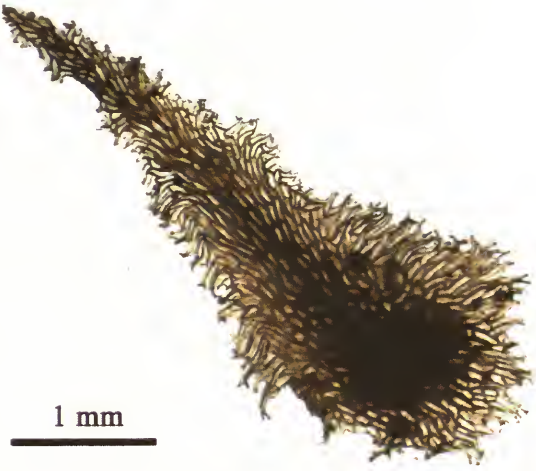

Figure 8: Peltate-based, lanceolate, fimbriate scale of abaxial laminar surface of *Pleopeltis ×cerro-altoensis* (from *Bizot 2905*). © Photo A. Bizot, 10/10/2014.

Figure 9: Sporangium and malformed spores of *Pleopeltis* ×*cerro-altoensis* (from *Bizot 2905*). © Photos A. Bizot, 8/10/2014 & 18/10/2014.

- Dried herbarium specimens issued from the cultivated plant at Mulhouse under number *CBM 03-301* :
  - *Boudrie 3966 (P)*
  - *Boudrie 4500 (P)*
  - *Danton Z(hs)1727 (private herb. Ph. Danton)*
- Other *Pleopeltis* taxa from Mexico, deposited in the personal herbarium of one of us (R. V.), but used, when freshly collected, for FCM analyses. Only one, *P. guttata*, is in cultivation in R.V.'s garden in Serskamp, near Ghent.
  - *Viane 11001: Pleopeltis guttata (Maxon) E.G. Andrews & Windham*
  - *Viane 11005: Pleopeltis polylepis (Roem. ex Kunze) T.Moore var. erythrolepis (Weath.) Wendt*
  - *Viane 11013: Pleopeltis polypodioides (L.) E.G.Andrews & Windham var. knoblochiana (Mickel) A.R.Sm. & Tejero*

Herbarium specimens of the respective private herbaria of A.B., Ph. D. and R. V., cited above, will be deposited in the future in official institutions.

To establish its characters and identity, several methods have been applied to the material mentioned above.

Micromorphological observations

Micromorphological observations were carried out by A.B. using a microscope Bresser triocular TRM-301 microscope at 400x to 1000x enlargements, equipped with a Bresser MicroCamLab-1 ocular camera. Fresh fronds from material in cultivation in the Botanical Conservatory of Mulhouse [*Bizot 2905*] were used.

- *Laminar scales* were observed (Figures 7 & 8) on fresh fronds. Rhizome scales were not microscopically observed. However, despite their more lanceolate shape than the laminar scales, they show the same clathrate pattern of cells.
- *Stomata*: Using tweezers, three epidermial slices were excised from the abaxial side of the lamina in the middle of a median pinna. The epidermis was observed in glycerin at 1000x enlargement. Eighty (80) stomata were measured.
- *Spores* were observed (Figure 9) in glycerin liquid at 1000x enlargement. They were collected from several (3-4) sori of three different fresh fronds as well as from more than two sori of a frond collected in the field [*Danton **B(652)619***].

Comparison of size of stomata of the Cerro Alto plant with stomata from plants of *P. macrocarpa* and *P. masafuerae* may reveal important information, but this investigation was impossible owing to the lack of adequate fresh material.

Measurements of rhizomes

The width (rhizomes are slightly flattened) and the thickness of rhizomes of the 10 specimens of *P. macrocarpa*, four specimens of *P. masafuerae*, and four specimens of the Cerro Alto plant were measured from dried herbarium specimens. Sectioned pieces of rhizomes were observed under a binocular and measured using a plastic ruler with the smallest calibrated unit being 0.5mm.

DNA content using FCM

A few fresh fronds (Botanical Conservatory of Mulhouse, France) of the *Pleopeltis* from Cerro Alto (in cultivation since 2002) were sent to the laboratory of Pteridology of Ghent University (Belgium) on 6th October 2014 for flow cytometric measurements (FCM). For this analysis, fresh samples were first chopped, using a sharp razor blade,

in a glass Petri dish containing nucleus-isolation buffer (2.1% [w/v] containing citric acid monohydrate, 0.5% [w/v] Tween 20, and distilled water; this suspension was then filtered through a 50-µm nylon mesh. The nuclear suspension was supplemented with DAPI reagent [6.5% $NaH_2PO_4.2H_2O$, 40µl DAPI stock solution (5mg DAPI/ml distilled water), distilled water], and each sample was measured three times. The samples were analysed using a flow cytometer (Ploidy Analyser, Partec GmbH, Münster, Germany), following the supplier's instructions. To calculate the relative nuclear DNA content, *Agave striata* (2n = 2x = 60, 2C = 7.8pg (Zonneveld et al., 2005) was used as an internal standard.

Fresh material of *P. macrocarpa* or *P. masafuerae* was unavailable, as the islands where they grow are difficult of access.

To establish ploidy levels, our FCM results were compared with those of some Mexican *Pleopeltis* taxa collected in Chihuahua: diploid *P. polylepis* (Roem. ex Kunze) T.Moore var. *erythrolepis* (Weath.) Wendt (*Viane 11005*); *P. guttata* (Maxon) E.G.Andrews & Windham (*Viane 11001*); and triploid *P. polypodioides* (L.) E.G.Andrews & Windham var. *knoblochiana* (Mickel) A.R.Sm. & Tejero (*Viane 11013*). To confirm the FCM results, a chromosome count was made for *P. guttata*, revealing it to be triploid (Van den heede, pers. comm.).

The three specimens from Cerro Alto submitted for FCM have been registered as "*Ph. Danton - Mulhouse* - 1" to "*Ph. Danton -Mulhouse*- 3", all sampled from the cultivated plant at Mulhouse under the number *CBM 03-301*.

RESULTS

Micromorphology, spores and stomata

Spores are either irregular in shape and in size or totally malformed (Figure 9), a condition typical for interspecific fern hybrids. Stomata range from 45 to 63µm long, with an average of 53µm and a standard deviation of 3.1µm.

Rhizome measurements

Taxon	Width (mm)			Thickness (mm)		
	min.	mean	max.	min.	mean	max.
Pleopeltis macrocarpa	1.2	**2.1**	2.5	0.7	**1.4**	2.0
Pleopletis masafuerae	3.0	**3.3**	4.0	1.8	**2.0**	2.4
Cerro Alto plant	2.3	**2.5**	2.8	1.5	**1.7**	2.1

Although the number of measurements is rather low, the values obtained for width and diameter of rhizomes from the Cerro Alto plant are intermediate between those of its putative parents, *P. macrocarpa* and *P. masafuerae*.

Flow cytometry

Comparison of FCM values of the Cerro Alto plant with those of Mexican *Pleopeltis* taxa shows that it is most probably tetraploid (4x). Polyploidy is known within *Pleopeltis*, and, within *P. macrocarpa*, three cytotypes, diploid, tetraploid and hexaploid, have been reported (Evans, 1963; Jarret et al., 1968; Walker, 1973; Wagner

& Wagner, 1975).

FCM results are presented in the table below:

Taxon	Voucher	$RC_{striata}$ *	Ploidy level
Pleopeltis polylepis var. *erythrolepis*	Viane 11005	1.56	2x
Pleopeltis guttata	Viane 11001	2.26	3x
Pleopeltis polypodioides var. *knoblochiana*	Viane 11013	2.54	3x
Pleopeltis ×*cerro-altoensis*	cult Mulhouse	3.06	4x

*$RC_{striata}$: average (based on 3 measurements) of relative DNA content compared with *Agave striata*.

From the value of 3.06 for $RC_{striata}$ for the new hybrid, we infer that it is tetraploid.

DISCUSSION

Interspecific hybrids are well known among species of *Pleopeltis*, as redefined by Smith & Tejero (2014), and are discussed there, as well as in several floras, e.g., Mickel & Beitel, 1987; Mickel & Smith, 2004. Such hybrids, mostly in the Neotropics but a few in southern Africa, have sometimes been treated under the hybrid genus, ×*Pleopodium*, because historically they were thought to involve species of two different genera, *Pleopeltis* and *Polypodium*. Our study of the Cerro Alto plant shows that it is a hybrid that clearly belongs to *Pleopeltis*.

Only two species of *Pleopeltis* occur in the Juan Fernández Archipelgo: *P. macrocarpa*, with entire, narrowly lanceolate laminae, and *P. masafuerae*, with deltate, pinnatisect laminae and undulate pinnae that are slightly decurrent onto the rachis. The latter species occurs on Alejandro Selkirk Island and on the coasts of Chile (Antofagasta province). Remarkably, *P. masafuerae* has never been found on Robinson Crusoe Island. However, due to the inaccessibility of large areas and infrequency of hybridization, it may have been overlooked.

The overall lanceolate laminar shape of the Cerro Alto plant is similar to that of *P. macrocarpa*, whereas its division, though less incised (pinnatilobed to pinnatifid) and with distinctly decurrent but less undulate pinnae, is more similar to *P. masafuerae*. In addition, we have compared the laminar morphologies of the Cerro Alto plant with the *Pleopeltis* hybrids described by Wagner & Wagner (1975) and Mickel & Beitel (1987), in particular between species with entire, lanceolate laminae, such as *P. macrocarpa* or *P. mexicana* (Fée) Mickel & Beitel, and a species with deltate pinnatisect laminae, such as *P. thyssanolepis* (A.Braun ex Klotzsch) E.G.Andrews & Windham that has laminae similar to those of *P. masafuerae*. Two hybrids, *P.* ×*leucospora* (Klotzsch) T.Moore and *P.* ×*tricholepis* (Mickel & Beitel) T.Moore, show a laminar morphology very similar to that of the Cerro Alto plant. An interesting similarity is the irregularity of the shape of the laminae and of the length of the pinnae, pointed out by Wagner (1962), that we notice also in our plant. However, the Cerro Alto plant differs from *P.* ×*leucospora* by a shorter stipe and a shorter terminal segment, and from *P.* ×*tricholepis* also by a shorter stipe, broader pinnae and a lamina reduced at its base.

These observations lead us to believe that *P. macrocarpa* and *P. masafuerae* are probably the parents of the Cerro Alto plant. As the hybrid is presumed tetraploid, based

on FCM values, we assume that tetraploid *P. macrocarpa* is involved in the combination, and that *P. masafuerae* is also tetraploid.

We conclude that the Cerro Alto plant is a hybrid new to the Juan Fernández Archipelago.

Pleopeltis ×cerro-altoensis Danton & Boudrie, **hyb. nov.**
Abortivis sporis, tetraploideum, hybridum filix est, repente rhizomate, 5-7 cm in longitudine fronde, angustissime alato atrobrunneo petiolo, appressis, fimbriatis peltatisque paleis penitus pinnatifido lanceolato limbo atque infra atrobrunnea et super viridiluteola rhachidi. A P. macrocarpa *pinno-pinnatifido limbo, et a* P. masafuerae *basi reducta lanceolato limbo praecipue differt.*

Types: CHILE. Archipel Juan Fernández, île Robinson Crusoe, Cerro Alto, Quebrada Puerto Inglés, rochers en falaise sur le versant est-sud-est du cerro, 300 m, 22 janvier 1999, *Danton B(652)619* [holotype: SGO (Figure 6); isotypes: P, private herb. P. Danton].

Description: Epilithic fern. *Rhizomes* creeping, 2.5-3 mm diam. (4-5 mm including the scales), branched, densely covered with oblanceolate, acute, clathrate, bicolorous scales, with dark centres and narrow, light brown, and finely dentate-fimbriate margins. *Fronds* monomorphic, 0.5-1 cm apart, 6-15 × 0.6-4 cm. *Stipes* 2-5 cm, about $^1/_3$ - ¼ as long as laminae, dark brown, with sparse, elongate scales. *Laminae* coriaceous, dark green, matt adaxially, light green to yellowish and slightly glaucous abaxially, narrowly elliptic to lanceolate, deeply pinnatifid, tapering proximally to reduced lobes, and below that long-decurrent (young fronds entire to sinuately lobed), distally ending in a long pointed to obtuse segment; laminar surfaces covered with numerous, scattered, peltate-based, appressed, bicolourous lanceolate scales with dentate or fimbriate margins, scales more abundant abaxially than adaxially. Dark brown rachis colour ending at the base of the apical segment abaxially, adaxially the colour ending at the first or second proximal pinnae and becoming green-yellowish distally. *Pinnae* 5-7 per side, triangular to oblong, 24-26 × 5.5-6 mm (for the longest ones), of variable length, oblique to rachis, alternate to more opposite in the distal half of the laminae, apices obtuse to rounded; laminar margins cartilaginous and slightly recurved. *Stomata* (45)-53.5-(63) µm long. *Sori* round to slightly oblong, ca. 0.3 cm diam., exindusiate, mixed with peltate scales, median on both sides of the costae or (in less divided blades) of the rachis. *Spores* whitish to yellowish, malformed and irregular in shape and in size, (53)-73.5-(90) µm long. Tetraploid (4*x*).

Etymology: The epithet of the taxon refers to the locality, Cerro Alto, where the plant was found.

Distribution and habitat: To our knowledge, endemic to Robinson Crusoe Island (Masatierra) in Juan Fernández Archipelago (Chile), on southern flanks of Cerro Alto. Plants grow in a limited population of four small, relatively close patches, on small rocky edges covered by thin soil, on subvertical unstable volcanic cliffs, exposed to the winds, at 300 m elevation (Figure 3). Due to its ability to revive, it can tolerate periods of drought.

Specimens examined (paratypes): CHILE. Juan Fernández Archipelago, Robinson Crusoe Island, Quebrada Puerto Inglés, rebords de rochers en falaise et balmes sur le versant est-sud-est du Cerro Alto, 300 m, M. Boudrie et session SBF, 20 février 2002, *Boudrie 3793* (P).

Specimens *ex horto* examined (paratypes): CHILE. Juan Fernández Archipelago, Robinson Crusoe Island, Puerto Inglés, rebords de rochers en falaise et balmes sur le versant est-sud-est du Cerro Alto, 300 m, *ex horto* Conservatoire Botanique de la ville de Mulhouse (Haut-Rhin, France), *M. Boudrie et J.-P. Reduron*, 22 août 2003, *Boudrie 3966* (P); *idem*, *Ph. Danton et M. Hildebrand*, 13 juin 2006, *Z(hs)1727* (private herb. Ph. Danton); *idem*, *M. Boudrie et J.-P. Reduron*, 5 octobre 2009, *Boudrie 4500* (P); *idem*, *M. Hildebrand*, 29 septembre 2014, *Bizot 2905* (private herb. A. Bizot), and *P. Danton - Mulhouse - 1, 2 & 3* (private herb. R. Viane).

IUCN Status: "CR"

Conservation ex situ: A plant was previously cultivated, from 1999 to 2004, in the garden of the Administration of the National Park, on Robinson Crusoe Island, but is now dead. Another specimen has been maintained and propagated in the Botanical Conservatory of the town of Mulhouse (France) since 2002.

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A CONTRIBUTION TO THE PHYLOGENY OF *DRYOPTERIS REMOTA* BY GENOTYPING OF A FRAGMENT OF THE NUCLEAR *PgiC* GENE

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ABSTRACT

Since its discovery by A. Braun in 1834, there has been speculation on the origins of *Dryopteris remota*. The present contribution suggests that none of the previous assumptions are correct. *Dryopteris remota* is triploid and of hybrid derivation. Its parental species are the sexual allotetraploid *D. carthusiana* and an as yet unidentified sexual diploid species, most likely identical with the unknown parent of the apomictic diploid *D. affinis*. These assertions are based on comparison of traits, including habit, apomixis, colour of insertions of pinnae of the species under examination, and fragment length variation of the nuclear *PgiC* gene.

INTRODUCTION

The phylogeny and taxonomic status of *Dryopteris remota* (A.Br.) Druce is probably one of the most controversially discussed of any Central European fern species. When Alexander Braun discovered the plant near Baden-Baden in 1834 (Baden-Württemberg/ S-Germany), he thought the new taxon to be a variation of *D. villarii* (Bellardii) Woynar ex Schinz & Tell. As such it was listed in 1843 by J. C. Döll in "Rheinische Flora". In his book "Betrachtungen über die Erscheinung der Verjüngung in der Natur" of 1851, Braun revised his view in a footnote and described the taxon as a hybrid of *D. filix-mas* (L.) Schott and *D. carthusiana* (Vill.) H.P.Fuchs or *D. dilatata* (Hoffm.) A.Gray. He nonetheless attributed species rank to this taxon by calling it "*Aspidium remotum*", i.e. in current nomenclature *D. remota*. Since then a substantial number of renowned fern experts have speculated on its origin and taxonomic classification. In 1973 Benl and Eschelmüller presented an exhaustive description of this continuing dispute among scientists.

Further confusion had arisen following the discovery of a fern hybrid near Lake Windermere in north-west England in the middle of the 19th century. The morphology of this hybrid resembled very much that of *D. remota,* but it had abortive spores. In 1859 A. Braun found a single plant of *D. remota* among a large population of *D. filix-mas* near Aachen (Nordrhein-Westfalen/ Germany), which led him to the conclusion that *D. remota* must be a variation of *D. filix-mas*. He did not realize that this plant was not *D. remota* but actually the same hybrid as that from Lake Windermere. He transplanted this hybrid to the Botanical Garden of Berlin and for approximatively 15 years he sent incorrectly labelled exsiccata from there to herbaria and to individual scientists before this mistake was discovered (Benl & Eschelmüller,

1973). Further examination of a frond sample in the herbarium at Bonn revealed that Braun's finding was the second record of the tetraploid hybrid *D. carthusiana* × *D. filix-mas,* as discovered earlier at Lake Windermere (Krause et al., 2001), and which has now been typified as *D.* × *brathaica* Fraser- Jenk. & Reichst. (1977). It is now possible to distinguish the two taxa through cytology (Manton, 1950), morphology (Fraser-Jenkins & Reichstein, 1984; Krause et al., 2001; Freigang et al., 2013), chemotaxonomy (Widén et al., 1976) and molecular genetics (Freigang et al., 2013).

Dryopteris remota is today recognised as a triploid species of hybrid derivation, but the dispute concerning its origin continues. From fundamental works by W. Döpp (1932; 1933; 1935; 1939; 1955) and I. Manton (1932; 1938; 1939; 1950), Döpp considered *D. remota* to be the hybrid of *D. dilatata* × *D. affinis* (Lowe) Fraser-Jenk. (Döpp, 1955). Reichstein, Fraser-Jenkins and others are of the opinion that the diploid apomictic *D. affinis* must be one of the parents due to the characteristics of *D. remota* (Fraser-Jenkins & Reichstein, 1984). In consequence the second parent would have to be a diploid sexual taxon that contributes the characteristics known from the *D. carthusiana* group to the genotype of *D. remota. Dryopteris expansa* (C.B.Presl) Fraser-Jenk. & Jermy was proposed (Widén et al., 1971, Fraser-Jenkins & Reichstein, 1984), even though chemo-taxonomic results did not match well (Widén et al., 1970; 1971; 1996). Furthermore, in an on-line discussion of the British Fern Group, Fraser-Jenkins (30 December 2008) reiterated doubts as to *D. expansa* being involved: "Although I agree that *D. expansa* is the most likely second ancestor of *D. remota*, it ought to have a ? along that line as it has never been proved in any way and the possibility of some other, even perhaps Asiatic species being involved still remains."

To shed light on the ongoing discussions, the nuclear *PgiC*-marker of many species of the genus *Dryopteris*, including *D. remota*, has been examined.

MATERIAL AND METHODS

Material

The samples used in the investigation are listed in Table 1. Herbarium vouchers, currently in the collectors' private herbaria will be deposited in JE.

Methods

Samples were analyzed at the nuclear *PgiC* gene (cytosolic 6-Phosphoglucose-isomerase), individual fragments were generated and their lengths compared. The intron fragment between exons 15 and 16 proved especially suitable for comparison as it shows a species specific length for a number of species of the genus *Dryopteris*, especially in the *D. carthusiana* group (Juslén et al., 2011, Freigang et al., 2013). For this fragment Ishikawa et al., 2002 constructed an 'EPIC'- primer pair (Exon Primed Intron Crossing), which offers almost general applicability. Due to a high frequency of poor PCR yields or complete PCR failures, the method described in Freigang et al. (2013) was partly replaced by the PCR-direct method of ThermoScientific (Phire Plant Direct PCR Kit, F-130WH). This method worked without DNA isolation and PCR yields were often higher and, according to the producer instructions the workflow consisted of only two steps.

1. A disc of 0.3-0.5 mm diameter was punched out from fresh or silica gel-dried leaf material and put into PCR-solution. 2, PCR was carried out according to the producer instructions. The uncleaned amplicon was genotyped in a sequencer (ABI 3500, Applied Biosystems or MegaBace Instrument, Healthcare). Employment of different

sequencers made transformation of results necessary (marked with an asterisk). The analysis of the generated data was first made with Fragment Profiler software (Healthcare), then later with GeneMapper software (Applied Biosystems).Sometimes the sequencing device produced a double peak in consecutive basepairs (bp). This could be an artefact or may be the result of PCR-conditions.

The relative height of signals within an electropherogram depended firstly on the number of sets of chromosomes causing the signal and, secondly, on the relative PCR yield of the 15/16-fragment of the existing alleles.

RESULTS

The genotypes of samples 1 to 24 are summarised in Table 2. Signals of electropherograms which were not separated are noted in the same table-field. Electropherograms of a subset of samples are shown in Figures 1-3 (underlined in Tables 1 and 2).

DISCUSSION

In the case of apomictic reproduction in ferns the sporophyte grows directly from a gametophyte with the unreduced number of chromosomes, and without gametes being involved. The sporophyte produces genetically uniform diplospores through a restitution nucleus; in the case of apomicts of *Dryopteris*, restitution occurs in the last mitotic cell division before the production of spore mother cells (Manton, 1950), resulting in the production of eight, rather than the usual 16 spore mother cells. Sporogenesis then results in the production of 32 (instead of the usual 64) spores, each with the same number of chromosomes as the sporophyte.

Fischer (1909; 1919) detected apomixis in *D. remota* and Manton demonstrated that it is a triploid taxon, with three different (non-homologous) chromosome sets (i.e. a heterotriploid fern) as in the occasional production of a spore mother cell of the '16 cell type', the chromosomes are unpaired (non-homologous), with 123 univalents (3x). The heterotriploid character is also shown by the electropherogram of *PgiC* 15/16 fragments of the species (Figure 1), where three distinct peaks are observed.The electropherogram shows the following: The genome of *D. remota* contains at least three chromosome sets possessing fragments of different length and therefore derived from different alleles. This supports the results of cytology that the species is heterotriploid. The two shorter fragments of 488 bp and 500 bp correspond to the results seen in *D. carthusiana* (Figure 2), an allotetraploid species; no other taxon of the European ferns of the genus *Dryopteris* examined up to now shows this pattern.

Comparison with *D. cristata* (L.) A.Gray (sexual allotetraploid) and *D. intermedia* (Mühl. ex Willd.) A.Gray (sexual diploid) reveals that the peak at 488 bp differs from that of *D. intermedia* (500 bp), and is generated by the chromosome set derived from the common parental taxon of *D. cristata* and *D. carthusiana*, referred to as *D. "semicristata"* (Sc) by Walker (1955), a diploid species as yet unknown. The signal at 500 bp in *D. remota* is identical with that of *D. intermedia* (I). The chromosome sets of *D. carthusiana* can be symbolized by ScScII. The assumption that the parental species of *D. carthusiana* are *D. intermedia* and *D. "semicristata"* is supported (Table 2) and confirms the result of Sessa et al. (2012). The results are also in agreement with chloroplast sequence data by Juslén et al. (2011), which showed that *D. carthusiana* and *D. remota* share a maternally inherited set of chromosomes, referred to as *"semicristata"*. Table 2 shows that the 15/16-fragment length of *D. expansa* is the same

Table 1: Genotyped samples (Nomenclature according Fraser-Jenkins & Reichstein, 1984 and Fraser-Jenkins, 2007). RF: numbers according to rasters (see Niklfeld, 1971). Abbreviations of the names of collectors: GZ (G. Zenner/ Kirn), HWB (H. W. Bennert/ Ennepetal), JF (J. Freigang/ Bergatreute), SJ (S. Jeßen/ Chemnitz), WB (W. Bujnoch, Trier).

Sample no. & species	Origin	Collector/ Determination/ Date	Internal Data
1. *Dryopteris aemula*	Portugal/ Azores/ São Miguel/ N-side and ridge at Pico da Vara, 1100 m MSL	GZ 28.5.2013 Dr 8	MPb1458, DE807, RUN32/2H
2. *D. affinis*	Portugal/ Madeira/ N Funchal/ c. 600 m S of restaurant Ribeiro Frio at the roadside EN 103	JF/GZ 25.8.2011 JFV1086	MPb1724, RUN45/7A
3. *D. affinis*	Germany/ RP/ Palatinate/ NE Wilgartswiesen, rock at Wiligartsburg, ca. 230 m MSL, RF 6713/32	WB/GZ 6.8.1996	DE032, RUN21/11B
4. *D. carthusiana*	Germany/ NRW/ Märkisches Land, SW Hagen/ near Hasper dam, RF 4603	HWB 27.05.2013	MPb1442, DE791, RUN32/1B
5. *D. carthusiana*	Germany/ NRW/ niederrheinisches Tiefland/ E Venlo/ Nettetal/ Poelvenn–Kuhlen/ RF 4603/23	N.Neikes 05.07.2009	MPb1482, DE812, RUN32/3E
6. *D. carthusiana*	Germany/ RP/ middle Mosel/ W Trier/ Sirzenicher Bachtal, RF 6205/23	WB 17.7.2005	DE083, RUN22/10B
7. *D. carthusiana*	Germany/ BW/ Oberschwaben/ NE Ravensburg/ SE Bergatreute/ near Kiliansweiher, RF 8124/32	JF 22.10.2006 JF 2006	DE385, RUN20/12A
8. *D. cristata*	Germany/ RP/ Eifel/ SSE Daun/ Schalkenmehrener Maar, RF 5807/13	WB 28.8.1984 GWBT2 spore cultured	DE024 RUN20/G11
9. *D. dilatata*	France/ Haut–Rhin/ Vosges/ S Col de la Schlucht/ Sentier des Roches/ NE "les Trois Four", 1050 m MSL, RF 7908/31	GZ/P.Holveck 27.8.2014 Dr 4	MPb1665, RUN40/11D
10. *D. expansa*	Germany/ BY/ upper Allgäu/ West–Grünten/ N Burgberg/ N „Weinhalde", northern slope, 780–795 m MSL, RF 8427/41	GZ 19.7.2004 Dr 20	MPb1229, DE695, GWBT156 RUN28/11E
11. *D. expansa*	Switzerland/ Wallis/ S Leukerbad/ Dala Valley/ lower Lochwald, 1260 m MSL, RF 9611/23	GZ 18.7.2000 Dr 1	MPb1230 DE696, GWBT182 RUN28/11F

12. *D. intermedia*	USA/ Vermont/ Rutland/ Leicester Hollow Trail/ NNE Brandon	HWB 15.8.2001 SJ-3476	DE368 RUN25/A11
13. *D. oreades*	France/ Hérault/ Haut Languedoc,/W Bédarieux/ N Colombières-s-Orb/ near "La Fage", c. 760 m MSL	GZ/WB 23.7.1994 Dr 1	DE067, GZ-58, RUN18/11C
14. *D. oreades*	Italy/ Toscana/ Appennino Tosco Emiliano/ NW Pistoia/ W Abetone/ W M Gomito/Val di Luce, c. 1480 m MSL	SJ/J.Müller 12.8. 1992 SJ-2226	DE288, RUN19/11B
15. *D. pallida* subsp. *pallida*	Italy/ Sicily/ Mount Etna above Zafferana	JF 1.1.2007 JFV542	MPb1562 DE861 RUN37/B12
16. *D. remota*	Germany/BW/ Northern–Schwarzwald/ SSE Bad Herrenalb/ valley of Rothenbach, 440 m MSL, RF 7216/21	GZ/WB 1.8.1990 spore cultured	FZ-90/7, GWBT 32, DE26, RUN19/12G
17. *D. remota*	Germany/ BY/ W Berchtesgaden/ Ramsau/ SW of Schwarzbachwacht, c. 900 m MSL, RF 8343/31	GZ/JF 5.8.2013 Dr 2,	MPb1516, DE 824, RUN35/11B
18. *D. remota*	Schwitzerland/ Berner Oberland/ Innertkirchen/ Urbachtal in Vordertalwald, RF 9315/12	SJ 21.7.1992 SJ-2270	MPb1235, DE 637, RUN28/ 11C
19. *D. remota*	Austria/ Salzburg/ Pinzgau/ E Rauris/ valley of Geißbach, c. 1060 m MSL, RF 8744/31	GZ 6.8.2014 Dr 10	MPb1650, RUN44/11H
20. *D. remota*	Romania/ S-Karpaten/ N- Făgăraş/ NW des Rifugio "Negoiu"/ SSE Porumbacu de Sus/ Şerbota Tal c. 1000 m MSL	SJ 5.8.1985 SJ-2560-2	MPb1236, DE638, RUN28/ 11D
21. *D. remota*	Germany/ BY/ West-Allgäu/ NW Scheidegg/ forest N "Lötz", c. 700 m MSL, RF 8425/11	JF 4.6.2011 JF-V1008	MPb1250, DE710, RUN28/12D
22. *D. remota*	North-Italy/ Prov. Varese /E Luino/ W Ponte Tresa/ Tresa-Valley near Biviglione, 250 m MSL, RF 0018/21	GZ/JF/SJ 15.6.2014 Dr 33	MPb1580, RUN40/9D
23. *D. remota*	Northeast-Turkey/ Rize/ E Trabzon, S Ardeşen, Cat - Çamlihemşin, c. 400m MSL	SJ-3026, TR-4011, CRFJ 4037, 27.08.1973	MPb1738, RUN47/A1
24. *D. villarii*	Bulgaria / North-Pirin/ E des Kameniski vrah/ NW des Vihren/ W Bansko, Bela-reka-Valley, 2150 m MSL	SJ 24.7.1981 SJ-335	MPb1452 DE801, RUN32/2C

Table 2: Genotyping results for the length of the 15/16 *PgiC* fragment of samples 1 to 24 in base pairs (bp). The sample numbers in Table 2 correspond with those in Table 1. The measurements in Table 2 indicate that the two shorter fragments of *D. remota* correspond with those of *D. carthusiana* and the third fragment of *D. remota* with that of *D. affinis* and *D. oreades*.

Species	Fragment length in base pairs (bp)					
1. *Dryopteris aemula*				525		
2*. *D. affinis* subsp. *affinis*					530	
3. *D. affinis* subsp. *affinis*					530	
4. *D. carthusiana*	487	499				
5. *D. carthusiana*	486	499 500				
6. *D. carthusiana*	486 487	498 499				
7. *D. carthusiana*	487	500				
8. *D. cristata*	487			521		
9. *D. dilatata*		499	517			
10. *D. expansa*		499 500				
11. *D. expansa*		499 500				
12. *D. intermedia*		500				
13. *D. oreades*					529 530	
14. *D. oreades*					530	
15. *D. pallida* subsp. *pallida*			516			
16. *D. remota*	487 488	499 500			530	
17. *D. remota*	487	500			531	
18. *D. remota*	487	499			530	
19*. *D. remota*	489	501			530	
20. *D. remota*	487	500			530	
21. *D. remota*	488	500			530	
22. *D. remota*	489	501			531	
23*. *D. remota*	489	501			531	
24. *D. villarii*						532 533

* Employment of different sequencers made transformation of results necessary.

as that of *D. intermedia*. As mentioned in the introduction, the involvement of *D. expansa* in the parentage of *D. remota* has been the subject of much discussion. But this scenario would entail that the second parent of *D. remota* must be a diploid apomictic taxon of the *D. affinis* agg. with signals at 488 bp and 530 bp. Despite study of many taxa of the *D. affinis* agg., this combination has never been found. The same applies to the combination of signals at 500 and 530 bp.

During the first half of the last century *D. remota* was thought to be a hybrid of *D. carthusiana* × *D. filix-mas*, and Döpp endeavoured to backcross *D. remota* with the supposed parental species. He was successful in hybridizing *D. remota* only with *D. carthusiana* (Döpp, 1935). This provides some support for the conclusion that *D. carthusiana* is one parent of *D. remota*.

Already H. Fischer pointed to the fact that such a hybrid should also emerge in nature and he mentioned that the pharmacist H.K. Woynar (Graz) allegedly found intermediate forms in mixed stands of the two species at different sites in Tirol. Since 2011 putative hybrids of *D. remota* × *D. carthusiana* have been found at various locations in the wild (Freigang et al., in prep.).

The remaining question relates to the unknown identity of the third signal in the electropherogram of *D. remota*, at 530 bp. The chromosome set causing this third signal at 530 bp would have to contribute two qualities to *D. remota* that are characteristic of it and cannot be derived from the genetic material of *D. carthusiana* - the obligatory apomixis and the blue-black colour at the insertion of the pinnae (Fraser-Jenkins & Reichstein, 1984). Both features also exist in the *D. affinis* agg., but not in that of *D. filix-mas*. Examination of the diploid taxon *D. affinis* (Figure 3) shows a single peak at 530 bp.

According to current opinion *D. affinis* is a diploid apomict fern that shows non-homology of the two chromosome sets in 16-celled sporangia (Fraser-Jenkins, 2007) and it is presumed to have been derived from a cross between the sexual diploid *D. oreades* Fomin (OO) and a sexual diploid taxon, not yet identified but referred to as *D. "semiaffinis"* (SaSa) (Krause, 1998). The *PgiC* 15/16 fragments of both taxa correspond to the third peak in *D. remota*. As *D. remota* is triploid and its genome

Figure 1. Electropherogram of *Dryopteris remota* (Schwarzwald, Smp.No. 16)

Figure 2. Electropherogram of *Dryopteris carthusiana* (Bergatreute, Smp. No. 7)

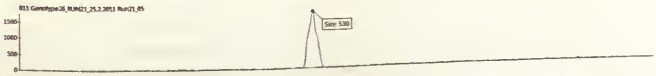

Figure 3. Electropherogram of *Dryopteris affinis* (Wilgartswiesen, Smp. No. 3)

contains two sets of chromosomes of allotetraploid *D. carthusiana*, *D. affinis* as the second parent is out of question, because as an apomictic taxon it would contribute both its chromosome sets to *D. remota*, resulting in a tetraploid taxon. This implies that the third peak (530 bp) in *D. remota* must be derived from a diploid sexual taxon with the 530 bp peak, and the only possible candidate from the results presented here are *D. oreades* or the missing diploid taxon *D. "semiaffinis"*.

Unfortunately the length of *PgiC* 15/16 fragments of *D. oreades* and *D. "semiaffinis"* cannot be distinguished with the method used here. Of these two, *D. "semiaffinis"* is the more likely parent; *D. oreades* does not exhibit blue-black insertions, nor apomixis. We are left to infer that both diploid *D. affinis* and triploid *D. remota* have inherited these characteristics from *D. "semiaffinis"*, and hence the phylogeny of *D. remota* could be analogous to that of *D. affinis,* as shown in Table 3.

All the evidence suggests that "*semiaffinis*" induces apomixis in its hybrids and species of hybrid derivation, in contrast to hybrids involving *D. oreades*. This is the case in all heterodiploid as well as heterotriploid taxa of the *D. affinis* complex, which are all fertile but to varying degrees. In hybrids of taxa of the *D. affinis* complex with *D. filix-mas*, apomixis is also expressed, despite that fact the "*semiaffinis*" chromosome set is only one of four or five sets in the nucleus. The hybrid of *D. carthusiana* and *D. "semiaffinis"* (Table 3) has three non-homologous sets of chromosomes and would be expected to be sterile, in contrast to *D. remota* which is fertile and can produce viable offspring in the wild. This case of *D. remota*, gives further support to the hypothesis that

Table 3. Diagram of the hypothetical genesis of *Dryopteris remota* and *D. affinis*
Abbreviations of chromosome sets: O = *oreades*, Sa = "*semiaffinis*", I = *intermedia*, Sc = "*semicristata*"

Parental Taxa	*D. oreades* homodiploid, sexual	*D. "semiaffinis"* homodiploid, sexual	*D. carthusiana* allotetraploid, sexual
Chromosome sets of a somatic cell	OO	SaSa	IScISc
Chromosome set(s) of a gamete	O	Sa	ISc
Hybridisation			
Primary hybrid	OSa heterodiploid, sterile		SaISc heterotriploid, sterile
Development of apomixis			
Progeny	*D. affinis*		*D. remota*
chromosome sets of a somatic cell	OSa heterodiploid, apomictic fertile		SaISc heterotriploid, apomictic fertile

the set of chromosomes from "*semiaffinis*" has the ability to transfer the qualities of dark insertion of pinnae, and apomixis coupled with fertility.

The distribution of *D. remota* lies between the Pyrenees and the Caucasus and thus falls within the distribution of *D. carthusiana* and *D. affinis* (Fraser-Jenkins & Reichstein, 1984). The genetic variability of *D. remota* was examined by the isozyme-method (Schneller & Holderegger, 1994) and also by RAPDs (Schneller et al., 1998). The first method did not reveal any genetic variability within or between populations, the second only little. The authors suggested that the species developed relatively recently and only once, presumably in Caucasus or the Mediterranean region, and spread over the now populated area by 'long-distance spore dispersal'. Since spores of apomicts produce sporophytes without the need for sexual fertilization, then an individual spore has the potential to give rise to a new individual or eventually even a new population.

During the past 180 years of discussion about the origin of *D. remota* other taxa have been proposed as possible parents, for example *D.villarii* (Braun in Döll, 1843), *D. dilatata* (Döpp, 1935; 1955), *D. aemula* (Aiton) O.Kuntze (Widén et al., 1971) or *D.pallida* subsp. *pallida* (Bory) Fomin and subsp. *raddeana* Fraser-Jenk. (Peroni et al., 1991). The results in Table 2 rule out almost all of these possibilities, with the exception of *D. pallida* subsp. *raddeana*, as samples of this taxon were not included in the study.

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SHORT NOTE

NOMENCLATURAL NOTE ON *HEMIONITIS ARIFOLIA* (PTERIDACEAE)

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The fern name *Hemionitis arifolia* (Burm.f.) T.Moore (Pteridaceae) is based on *Asplenium arifolium* Burm.f., first proposed by Burmann in 1768. Morton (1974: 316) found a sheet in the Geneva Herbarium (G, barcode G00360112, Figure 1) carrying two specimens and labeled as "Typus". Alston in August, 1952, had determined these specimens as juvenile *Acrostichum aureum* L., not *Asplenium arifolium*. As the type belonged to another taxon Morton (1974) suggested the next available synonym *Hemionitis cordata* Roxb. ex Hook. & Grev. as the correct name for this taxon. The sheet carries Burmann's annotation "*Arifolium*. Asplenium Arifolio Petiv Tab 50 Fig 12" indicating that Burmann saw these specimens (Figure 1). But in diagnosis he mentioned "simple ovate auriculate sinuous frond" and these specimens showing non-auriculate, non-sinuous frond (Figure 1), and not the basis of description or diagnosis validating the name (McNeill et al. 2012, Art. 9.3). Thus these specimens are not Types and on that basis the name *Asplenium arifolium* cannot be reduced as synonym of *Acrostichum aureum*. *Hemionitis arifolia* is the correct name for *Hemionitis cordata*.

In the protologue Burmann mentioned Petiver's (1702, t. 50, f. 12) and van Rhede tot Drakestein's (1692-1703, t. 10, Fig. 2zzz) figures showing auriculate sinuous fronds and his diagnosis was based on these figures. Although Morton (1974) used the term "Type" for the specimen in G (Figure 1), he did not accept it, as is clear from his comment "If this really is the type…,"; it is not acceptable as lectotype (McNeill et al. 2012, Art. 7.10). Here I select van Rhede tot Drakestein's figure from *Hortus Indicus Malabaricus* (1703) as Lectotype. But, as no collection data is available for the specimen on which this drawing was based (Johnston 1970; Wagenitz 1978; McNeill et al. 2012, Rec. 8A.1 & 8A.2) an epitype is selected.

TYPIFICATION

Asplenium arifolium Burm. f., Fl. Ind. 231. 1768 — *Hemionitis arifolia* (Burm. f.) T. Moore, Index Fil. 114. 1859. — *Parahemionitis arifolia* (Burm. f.) Panigrahi, Amer. Fern J. 83(3): 90. 1993.

Lectotype (designated here): van Rhede tot Drakestein, Hortus Indicus Malabaricus 12: 21, t. 10, 1703 (Figure 2).

Epitype (designated here): India. West Bengal: Burdwan, Kanchannagar, near bridge above canal, 30 m, 21 Nov 2007, *J. Mazumdar 59* (CAL Figure 3).

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Figure 1. Type sheet of *Asplenium arifolium* Burm.f. from G (barcode G00360112). Image © Conservatoire et jardin botaniques de la Ville de Genève, Switzerland, reproduced with permission.

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Figure 2. Lectotype of *Asplenium arifolium* Burm.f.: Tab. 10 from van Rhede tot Drakestein's Hortus Indicus Malabaricus.

Figure 3. Epitype of *Asplenium arifolium* Burm.f. (J. Mazumdar 59) (CAL).

SHORT NOTE

CONFIRMATION OF *LOXOGRAMME LANKOKIENSIS* (POLYPODIACEAE) IN INDIA

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The small epiphytic fern *Loxogramme lankokiensis* (Rosenst.) C.Chr. was first reported to occur in India from the Tirap District, Arunachal Pradesh, by Singh & Panigrahi (1987). Later Singh & Panigrahi (2005) suspected that an earlier record (Bhargavan & Joseph 1978) of *L. grammitoides* (Baker) C.Chr., from Arunachal Pradesh, Lohit District, may also represent *L. lankokiensis* and not *L. grammitoides* (Baker) C.Chr. However, they were unable to locate the specimen at CAL and could not make a definite decision (Singh & Panigrahi 2005). During the taxonomic study of ferns of Eastern India, I found this specimen of *Loxogramme lankokiensis*: INDIA. Arunachal Pradesh: Lohit District (N.E.F.A.), forest around Hayuliang along Dalai river, *J. Joseph 48934* (originally from CAL, now at BURD; Figures 1A–C).

In recent revisions Fraser-Jenkins (2008, 2012) followed the opinion of Dr. M.G. Price (University of Michigan, Michigan) and claimed that the report of *L. lankokiensis* from India was based on the misidentification of small plants of *L. grammitoides*. Close examination of *J. Joseph 48934* showed features that matched with characters of *L. lankokiensis*as described in literature (Singh & Panigrahi 2005, Zhang et al. 2013). *Loxogramme lankokiensis* has dimorphic fronds (Figure 1C) and rhizome scales with entire margins whereas *L. grammitoides* has mostly monomorphic fronds and rhizome scales with slightly dentate margins, according to Zhang et al. 2013. This specimen (*J. Joseph 48934*) confirms the earlier report of *L. lankokiensis* in India, Lohit District, Arunachal Pradesh by Singh & Panigrahi (2005), in addition to China, Thailand and Vietnam.

Selected digitized specimens examined— *Loxogramme lankokiensis*: VIETNAM. Lankok ["Roches moussues du Mont Bavi, prèt de la vallée de Lankok"], 12 Jun. 1888, *B. Balansa 1934* (Isotypes B-20 0089150, BR-0000006979492, BM-001039924, BM-001039925, P-01356591); Tonkin: Cao-Bang, *D. Billet s.n.*, *Herb. H. Christ* (P-01356590). THAILAND. Udawn: Phu (Mt.) Luang, about 900 m, 11 Jan. 1966, *E. Hennipman 3631A* (P-01353469).— *Loxogramme grammitoides*: CHINA. S. Wushan, West Hupeh, *E.H. Wilson 620* (Isotypes B-20 0089149, US00067489, BM-001039922).

ACKNOWLEDGEMENTS

I sincerely thank the Director, Botanical Survey of India and Prof. R. Mukhopadhyay, Pteridology Section, Department of Botany, The University of Burdwan for kindly providing opportunity to study the specimen and Dr M. Lehnert, University Bonn, Germany for constructive suggestions.

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Figure 1. A.–C. *Loxogramme lankokiensis* (J. Joseph 48934 CAL): **A.** & **B.** Specimen from Arunachal Pradesh, India, **C.** Part of plant showing distinct fertile and sterile fronds.

SINGH, S. & PANIGRAHI, G. 2005. *Ferns and Fern-allies of Arunachal Pradesh (Adiantaceae to Nephrolepidaceae)*. Vol. 1. Bishen Singh Mahendra Pal Singh, Dehra Dun, 426 pp.

ZHANG, X.C., LU, S.G., LIN, Y.X., QI, X.P., MOORE, S., XING, F.W., WANG, F.G., HOVENKAMP, P.H., GILBERT, M.G., NOOTEBOOM, H.P., PARRIS, B.S., HAUFLER, C., KATO, M. & SMITH, A.R. 2013. Polypodiaceae. 758-850 pp., in WU, Z.Y., RAVEN, P.H. & HONG, D.Y. (eds), *Flora of China*, Vol. 2-3 (Pteridophytes). (http://flora.huh.harvard.edu/china/mss/volume02/Flora_of_China_Volume_2_3_Polypodiaceae.pdf)

SHORT NOTE

THREE NEW COMBINATIONS IN *CTENOPTERELLA* (POLYPODIACEAE)

Three new combinations are proposed for *Ctenopterella* (Polypodiaceae) in preparation for a monograph of the genus.

Ctenopterella lasiostipes *(Mett.) Parris* **comb. nov.**
Based on *Polypodium lasiostipes* Mett., Ann. Sci. Nat. 4, 15: 76-77 (1861). Type: New Caledonia, Poila, *Vieillard* 1601 (lectotype P, chosen by Brownlie, Fl. Nouv.-Caléd. 3: 274 (1969); isolectotype NSW!). **Synonym:** *Ctenopteris lasiostipes* (Mett.) Brownlie, Fl. Nouv.-Caléd. 3, Pteridophytes: 273 (1969).

Ctenopterella thwaitesii *(Beddome) Parris* **comb. nov.**
Based on *Polypodium thwaitesii* Beddome, Ferns Brit. India: t. 188 (1866). Type: Ceylon, *Thwaites C. P.* 3900 (lectotype K!, chosen by Parris, Fern Gaz. 16(5): 243 (2002); isolectotypes BM!, CAL 20454 images seen, CGE!, E 00195204!, GH!, K!, P 02141982!, PDA!). **Synonym:** *Ctenopteris thwaitesii* (Beddome) Sledge, Bull. B.M. (Nat. Hist.) Bot. 2(5): 155 (1960).

Ctenopterella undosa *(Baker) Parris* **comb. nov**.
Based on *Polypodium undosum* Baker, J. Bot. 28: 108 (1890). Type: New Guinea, Mt Musgrave, 1889, *MacGregor* 61 (holotype K 000507064!; isotypes MEL!). **Synonyms:** *Ctenopteris undosa* (Baker) Copel., Philipp. J. Sci. 81(2): 103 (1953); *Polypodium pycnophyllum* C.Chr., Brittonia 2(4): 308 (1937). Type: Papua, Central Division, Mt Albert Edward, 3680 m alt., 5 July 1933, *Brass* 4421 (holotype BM; isotypes BRI!, MICH 1115954!, NY) (**synon. nov.**), *Ctenopteris pycnophylla* (C.Chr.) Copel., Philipp. J. Sci. 81(2): 102 (1953).

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BOOK REVIEW

Farne als Lebensleidenschaft – Festschrift für H. Wilfried Bennert anlässlich seines 65.Geburtstages. Berichte des Instituts für Landschafts- und Pflanzenökologie der Universität Hohenheim. Beiheft 22. 2013. 238pp. 21.0 x 29.7cm (in German).

Available from A. Kleinsteuber, Weißdornweg 35, D-76149, Karlsruhe, Germany. email: kleinsteuber-books@kabelbw.de

This festschrift for Wilfried Bennert on the occasion of his 65th birthday is a collection of 11 papers gathered together by Karsten Horn and illustrated with a series of etchings of ferns by Reinhard Böcker. The subjects of the articles reflect Bennert's wide interests in pteridology and ecology. The volume starts with a description of his life and career in science, the Bochum days, and how he fell under the spell of ferns, horsetails and clubmosses. He carried out fieldwork not only in Europe but also in California, Iceland, Southeast Asia and Russia, and Horn describes the work that culminated in one of his major fern projects, the publication of *Die seltenen und gefährdeten Farnpflanzen Deutschlands* in 1999. This is followed by a full list of his publications.

The subjects and authors of the other papers are summarised below.

Reinhard Bornkamm describes the ferns, horsetails, *Ephedra* and gymnosperms of Egypt and their role in plant communities. Herbert Sukopp looks at three ferns in C19th horticulture around Berlin. Andreas Sarazin et al. describe new records of neophyte fern taxa that have been discovered in Northwest Germany, including species of *Adiantum*, *Cyrtomium* and *Pteris*. Böcker describes the historical and present distributions of ferns, lycophytes and horsetails from the Stuttgart area, and assesses the impact of human activity. Marcus Lubienski provides photographs, detailed descriptions and distributional data for all known hybrids of *Equisetum* in Europe, and their morphological variability is discussed. Caroline Van den heede and Ronnie Viane report the discovery of *Asplenium lolegnamense* and *A.×chasmophilum* and discuss their origins. Wolfgang Jäger reports the occurrence of ascomycete fungi on ferns in the Bergisches Land, Northrhine-Westphalia. Karsten Horn has published an account of the life and scientific output of K.A.J. Milde, a great pteridologist of C19th, with illustrations from some of his significant monographs. Henning Haeupler reviews and clarifies the work on life forms to describe vegetation, first introduced by Humboldt in 1806 and extended by Raunkiaer in 1907. The volume concludes with plant colonisation in shallow waters - plants of the littoral zone, by Klaus Kaplan.

Wilfried Bennert has been a member of BPS since 1975, and is active in the Group of European Pteridologists.

M. Gibby

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STEVENSON, D.W. & LOCONTE, H. 1996. Ordinal and familial relationships of pteridophyte genera. In: CAMUS, J.M., GIBBY, M. & JOHNS, R.J. (Eds) Pteridology in perspective, pp. 435-467. Royal Botanic Gardens, Kew.

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